

Characterization of VAMP isoforms in 3T3-L1 adipocytes: implications for GLUT4 trafficking

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ABSTRACT The fusion of GLUT4-containing vesicles with the plasma membrane of adipocytes is a key facet of insulin action. This process is mediated by the formation of functional soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes between the plasma membrane t-SNARE complex and the vesicle v-SNARE or VAMP. The t-SNARE complex consists of Syntaxin4 and SNAP23, and whereas many studies identify VAMP2 as the v-SNARE, others suggest that either VAMP3 or VAMP8 may also fulfil this role. Here we characterized the levels of expression, distribution, and association of all the VAMPs expressed in 3T3-L1 adipocytes to provide the first systematic analysis of all members of this protein family for any cell type. Despite our finding that all VAMP isoforms form SDS-resistant SNARE complexes with Syntaxin4/SNAP23 *in vitro*, a combination of levels of expression (which vary by >30-fold), subcellular distribution, and coimmunoprecipitation analyses lead us to propose that VAMP2 is the major v-SNARE involved in GLUT4 trafficking to the surface of 3T3-L1 adipocytes.

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INTRODUCTION

Insulin stimulates glucose transport in adipose and muscle tissue by inducing the movement ("translocation") of intracellular vesicles containing the glucose transporter, GLUT4, to the cell surface (Leto and Saltiel, 2012). Once at the plasma membrane, these vesicles dock and fuse, resulting in increased levels of functional glucose transporters at the cell surface and thus elevated levels of glucose transport into the cell (Bryant *et al.*, 2002). This translocation mechanism appears to be defective in individuals with type 2 diabetes or insulin resistance, and thus much effort has been focused on defining the molecular basis of this key action of insulin (Rizza and Butler, 1990; Shepherd and Kahn, 1999).

In the absence of insulin, GLUT4 is effectively sequestered inside the cell. This is achieved via two interrelated endosomal trafficking

cycles that culminate in a significant fraction of GLUT4 populating specialized GLUT4-storage vesicles (GSVs; Bryant *et al.*, 2002). Defining how these GSVs dock and fuse with the cell surface has focused attention on the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery for membrane fusion, as SNARE proteins are involved in all membrane-trafficking steps in eukaryotes. There is agreement that the plasma membrane-localized target (t)-SNARE complex comprises Syntaxin4 and SNAP23, but the identity of the vesicle (v)-SNARE involved in GSV fusion with the cell surface has been more controversial (Cheatham *et al.*, 1996; Zhao *et al.*, 2009; Bryant and Gould, 2011). Multiple v-SNAREs have been identified in GSVs, including vesicle-associated membrane protein 2 (VAMP2), VAMP3, and VAMP8 (Volchuk *et al.*, 1995; Larance *et al.*, 2005). These v-SNAREs have all been shown to play a role in exocytosis in other cell types and in each case do so via interaction with Syntaxin4/SNAP23 (Bajno *et al.*, 2000; Schoch *et al.*, 2001; Polgar *et al.*, 2002; Cosen-Binker *et al.*, 2008). The majority of studies of GSV exocytosis in adipocytes have focused on VAMP2, and studies using small interfering RNA (siRNA; Williams and Pessin, 2008; Kawaguchi *et al.*, 2010), dominant-negative inhibitory fragment expression (Millar *et al.*, 1999), or peptide interference (Martin *et al.*, 1998) have all supported a role for VAMP2 in insulin-stimulated glucose transport/GLUT4 translocation. However, data using toxin-specific cleavage of VAMP2 have been more controversial, with some studies reporting significant inhibition of

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Abbreviations used: GSV, Glut4-storage vesicles; PM, plasma membrane; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein.

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insulin-stimulated Glut4 translocation (Randhawa *et al.*, 2000) and others not (Hajdich *et al.*, 1997).

A study using knockout mice and toxin-based approaches to interrogate VAMP function demonstrated that simultaneous disruption of VAMPs 2, 3, and 8 was required to completely block insulin-stimulated GLUT4 insertion into the plasma membrane of adipocytes; this defect could be rescued by reexpression of VAMP2, VAMP3, or VAMP8 alone, a result interpreted to imply plasticity in the requirement for v-SNARE proteins in GLUT4 trafficking to the plasma membrane (Zhao *et al.*, 2009). This study appears contradictory to others, in which disruption of VAMP2 function alone was sufficient to impair GLUT4 translocation (Martin *et al.*, 1998; Millar *et al.*, 1999; Williams and Pessin, 2008; Kawaguchi *et al.*, 2010; Bryant and Gould, 2011). There are several possible explanations for these apparent discrepancies; key among these is that the absolute levels of the different VAMP isoforms when reexpressed in adipocytes are not known. This is important, as GLUT4 populates multiple intracellular compartments, and this depletion or inhibition of one arm of GLUT4 traffic (e.g., by siRNA or toxin treatment) may drive GLUT4 along another route to the cell surface. Hence systematic analysis of levels of expression, function, and distribution and the effect of insulin is warranted. Here we report such an analysis.

We show that all VAMP isoforms are capable of forming an SDS-resistant SNARE complex with Syntaxin4/SNAP23 *in vitro*. However, we found that VAMP isoforms differ widely in their levels of expression in 3T3-L1 adipocytes. VAMP3 and VAMP8 are expressed approximately twofold and approximately fourfold higher than VAMP2, respectively, but unlike VAMPs 2 and 3, VAMP8 levels do not increase upon differentiation of 3T3-L1 fibroblasts to adipocytes (a key hallmark of the GLUT4 trafficking machinery; El-Jack *et al.*, 1999). VAMP2 and VAMP3 both exhibit insulin-stimulated translocation to the cell surface, whereas VAMP8 does not. We also show that VAMP2 is localized within a GSV-enriched fraction to a far greater degree than VAMP3. Such data support the notion that VAMP2 is the major v-SNARE involved in GLUT4 trafficking from GSVs to the plasma membrane. Consistent with this, we show that immunoprecipitation of Syntaxin4 coprecipitates VAMP2 and VAMP5 but none of the other VAMP isoforms. Our data therefore support the notion that VAMP2 is the major v-SNARE involved in GLUT4 trafficking to the surface of 3T3-L1 adipocytes.

RESULTS

All VAMP isoforms can form SDS-resistant SNARE complexes with Syntaxin4/SNAP23

A crucial facet of the debate regarding which VAMP isoform(s) might be key for insulin-regulated GLUT4 delivery to the cell surface is whether these different VAMP isoforms form SNARE complexes with Syntaxin4 and SNAP23. Although this has been shown for some isoforms, (specifically VAMPs 2, 3, and 8; Polgar *et al.*, 2002), it is not clear how specific this is. To address this, we took advantage of the ability of SNAREs to form SDS-resistant ternary complexes. As shown in Figure 1, all of the VAMPs were capable of forming a ternary complex, consistent with other studies of Syntaxin1A, confirming that the interactions between t-SNAREs and v-SNAREs *in vitro* are promiscuous (Yang *et al.*, 1999). Such data are consistent with the notion that any of the VAMPs could, in principle, mediate fusion with the plasma membrane.

VAMPs are selectively up-regulated during differentiation

Many studies have shown that key regulatory molecules involved in insulin-stimulated GLUT4 trafficking are up-regulated upon differentiation of 3T3-L1 fibroblasts into adipocytes; these include GLUT4,

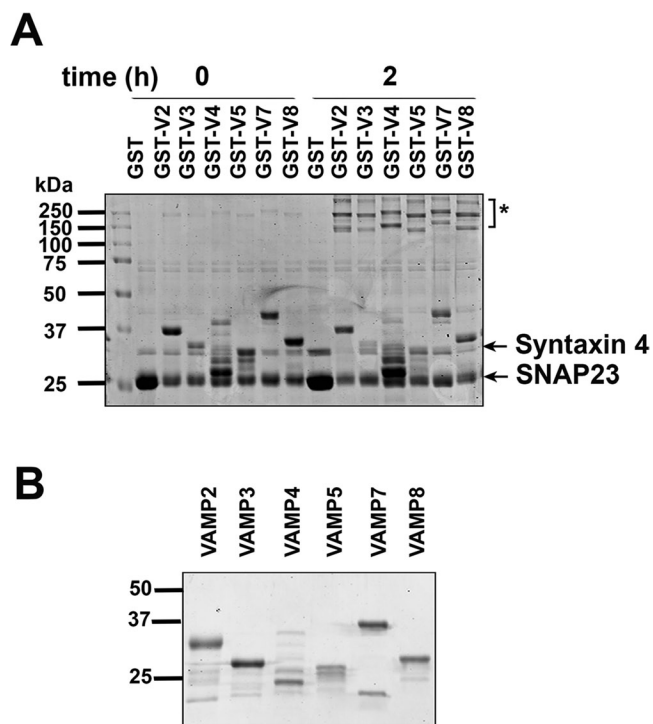


FIGURE 1: All VAMP isoforms form SDS-resistant complexes with Syntaxin4 and SNAP23. (A) Equimolar (~4 μ M) amounts of His-SNAP23, Syntaxin4, and GST-tagged VAMP (or GST alone) were combined, immediately mixed with 5 \times LSB, and heated to 95°C or incubated for 2 h at 4°C with rotation before being mixed with 5 \times LSB and heated to 95°C. Samples were subjected to SDS-PAGE, followed by staining with Coomassie brilliant blue. Data from a typical experiment repeated three times; molecular marker sizes are shown in kilodaltons. Molecular weights: His-SNAP23, ~25 kDa; Syntaxin4, ~32 kDa. The SNARE complex runs >110 kDa and is indicated by an asterisk. (B) Input VAMP isoforms used in the assay in A. A 1-mg amount of each purified recombinant VAMP isoform was subjected to SDS-PAGE, and protein was stained with Coomassie brilliant blue; molecular marker sizes are shown in kilodaltons.

VAMP2, Syntaxin16, and insulin-responsive aminopeptidase (El-Jack *et al.*, 1999; Perera *et al.*, 2003; Shewan *et al.*, 2003). We examined whether the levels of VAMP isoforms exhibited similar increases. Figure 2 shows an analysis of three different batches of 3T3-L1 fibroblasts and adipocytes immunoblotted for the different VAMPs. In each case, total cell numbers were estimated by counting propidium iodide-stained nuclei, and each lane on the blot shows membranes from an equivalent number of cells. Levels of VAMPs 2–4 all increased significantly during differentiation, but levels of VAMPs 5, 7, and 8 did not. Of note, under the conditions used for immunoblotting, the antibodies used were specific for the isoform indicated (unpublished data).

It is important to determine the absolute levels of expression of each of the VAMP isoforms, and we used quantitative immunoblotting with recombinant protein standards to achieve this (Hickson *et al.*, 2000). Total membrane fractions were prepared as described, and known amounts of membrane protein were separated on SDS-PAGE gels alongside known amounts of recombinant VAMP. Comparison of the immunoblot signals from the total membrane sample can thus be compared with known standards to allow estimation of the total amount of VAMP-x present in the membrane sample studied and then from a given number of cells, as

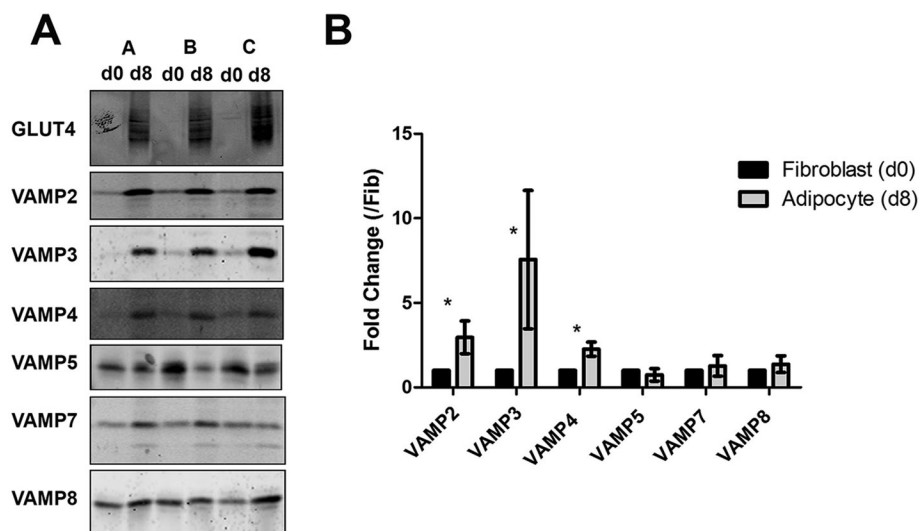


FIGURE 2: Selective VAMP isoforms are up-regulated during differentiation of 3T3-L1 adipocytes. (A) Equal numbers of fibroblast and adipocyte cells, quantified using propidium iodide staining of nuclei, were homogenized and subjected to centrifugation at $95,000 \times g$ for 1 h to pellet to the total membranes as described. Samples representing equal numbers of cells from day 0 (fibroblasts; d0) or day 8 (adipocytes; d8) were then subjected to SDS-PAGE, followed by immunoblotting with anti-GLUT4 and anti-VAMP antibodies as indicated. Three batches of membranes are displayed on each gel (A–C); d0, fibroblast. (B) The changes in VAMP levels upon differentiation were quantified. Values represent the means of three separate experiments in which fold changes compared with fibroblasts are shown for each VAMP member; values were compared using Student's *t* test, **p* < 0.05.

the latter was determined in parallel using propidium iodide staining of nuclei. In our hands, a 10-cm dish of 3T3-L1 adipocytes contained 10^7 cells and gave ~ 2.5 mg of protein (see legend to Figure 3). This relies on the antibodies in question cross-reacting with endogenous VAMPs to the same extent as is observed for purified recombinant proteins; although we have no reason to believe that this is not the case, the data should be interpreted with this caveat in mind. Representative examples of such analysis are shown in Figure 3, with the data for all VAMPs shown in Figure 3B. These data reveal wide differences in the levels of expression of the different VAMPs, with VAMPs 5 and 7 expressed at very low levels ($< 1 \times 10^5$ copies/cell). VAMP4 and VAMP2 were expressed at moderate levels (4.8×10^5 and 8.6×10^5 copies/cell, respectively). By contrast, VAMP3 and VAMP8 were highly expressed (1.5×10^6 and 3.5×10^6 copies/cell, respectively).

VAMP isoforms are differentially distributed

The data just described are important for comparing total cellular amounts, but they do not provide insight into the potential local concentration of VAMP isoforms among different cellular compartments; this is important, as such local concentrations could be significantly higher. With this in mind, we examined the distribution of the different VAMP isoforms among subcellular fractions comprising plasma membranes (PMs), light density membranes (LDMs; the location of the majority of GLUT4), and heavy membrane fractions (HDMs; Supplemental Figure S1 quantifies marker protein expression in these fractions to verify the effectiveness of the fractionation). In the absence of insulin, both VAMP2 and VAMP3 were predominantly ($> 60\%$) localized to the LDM fraction, consistent with previous studies of these VAMPs. By contrast, the remaining VAMPs exhibited broadly similar levels in both PM and LDM fractions (Figure 4A). Striking differences were observed when the effects of insulin on distribution were examined (quantified in Figure 4B). Consistent

with previous work (Cheatham *et al.*, 1996), insulin induced redistribution of VAMP2 and VAMP3 to the plasma membrane. Plasma membrane levels of the other VAMP isoforms did not increase in response to insulin (note that a trend toward increased levels of VAMP5 is evident in these data, but low levels of expression made this difficult to quantify accurately). Decreased levels in the LDM were observed for VAMP8, but increases in PM or HDM fractions did not reach statistical significance.

GLUT4 within the LDM fraction of adipocytes is known to be present in both early/recycling endosomes and GSVs. Iodixanol gradient analysis been used to resolve these fractions (Hashiramoto and James, 2000), allowing comparison of the VAMP distribution among these fractions to be determined (Figure 5A). Consistent with previous work, we find that VAMP2 is present within both fractions approximately equally (Martin *et al.*, 1996; Hashiramoto and James, 2000; Maier and Gould, 2000); similar distributions were observed for VAMP5 and VAMP8. VAMP3 is predominantly localized to the endosomal/recycling fractions ($> 75\%$ in the endocytic recycling compartment [ERC]; Figure 5B), also consistent with previous studies (Volchuk *et al.*, 1995; Martin *et al.*, 1998).

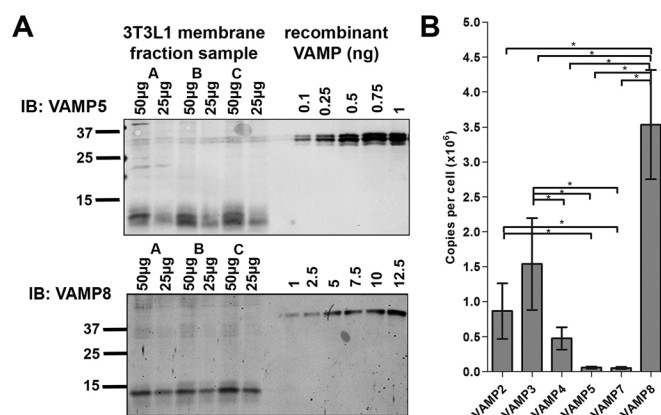


FIGURE 3: Quantification of VAMP levels using quantitative immunoblotting. Total membrane fractions from day 8 adipocytes were analyzed by immunoblotting alongside known amounts of purified recombinant VAMP (Figure 1B). Two examples, VAMP3 and VAMP8; each immunoblot contains membrane fractions (25 and 50 μ g) from three separate batches of cells (batches A–C). Data from a typical experiment. Depending on the VAMP being analyzed, a range of different protein loads was always used on multiple blots to ensure that detection was on the linear range of the response. (B) Quantification of these experiments. In our hands, one 10-cm dish contained 10^7 cells and 2.5 mg of membrane protein (Biber and Lienhard, 1986; Hickson *et al.*, 2000). For each batch of cells, we assayed the total membrane protein recovered and related this to the number of cells quantified using propidium iodide staining as outlined in the legend to Figure 2. The data shown are the mean and SD values obtained from three separate batches of 3T3-L1 adipocyte membranes; asterisk indicates a significant change, *p* < 0.05.

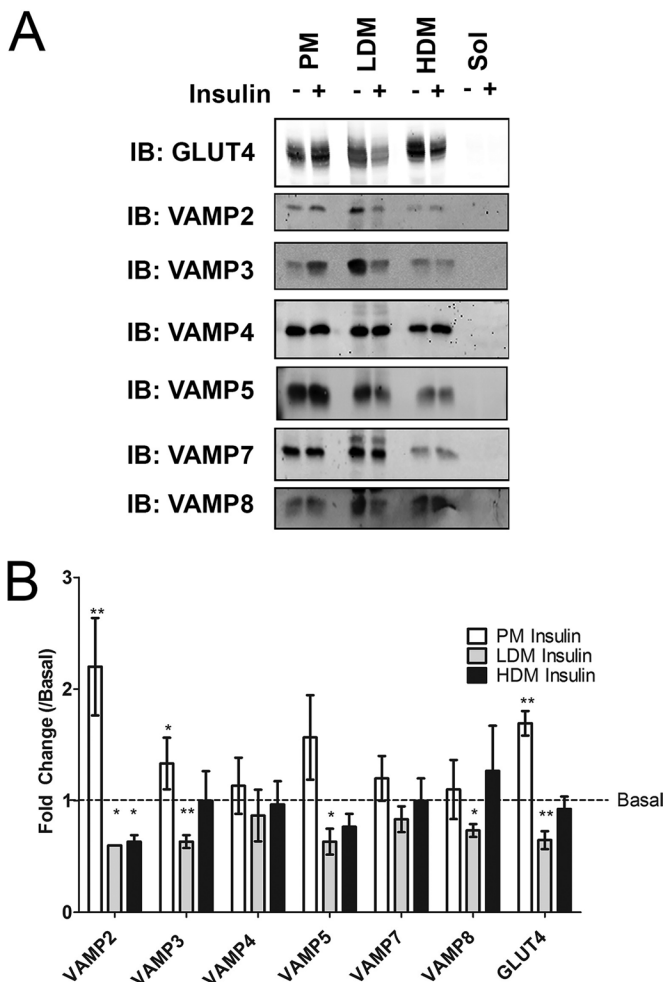


FIGURE 4: Subcellular distribution of VAMP isoforms in basal and insulin-treated adipocytes. 3T3 L1 adipocytes were treated with (+) or without (–) 1 μ M insulin for 20 min. Subcellular fractionation was performed as described generating PM, LDM, HDM, and soluble fractions (Sol; see also Supplemental Figure S1). Fractions were resuspended in equal volumes of 1 \times LSB and subjected to SDS–PAGE and immunoblotting with the indicated antibodies. (A) Representative immunoblots, in each case repeated with a minimum of three separate platings of cells. (B) Quantification of the changes in the distribution of each VAMP isoform in each fraction in response to insulin expressed relative to the basal levels in each fraction. Values represent means \pm SD of three separate experiments; fold change compared with basal values were compared using Student's *t* test, **p* < 0.05, ***p* < 0.01.

Similarly, VAMP4 and VAMP7 are found at somewhat greater levels in the ERC fractions.

Syntaxin4 coimmunoprecipitates VAMP2

To examine interactions of the VAMP family members with Syntaxin4 *in vivo*, we immunoprecipitated Syntaxin4 from basal and insulin-stimulated adipocytes and probed for coimmunoprecipitation of VAMP isoforms. We observed Syntaxin4/VAMP2 coimmunoprecipitation in insulin-stimulated cells and Syntaxin4/VAMP5 interactions in basal and insulin-stimulated conditions (the latter is consistent with data from cardiomyocytes; Schwenk *et al.*, 2010) but could detect no other VAMP isoform in Syntaxin4 immunoprecipitates under either condition (Figure 6). By contrast, VAMP4 was readily coimmunoprecipitated by anti-Syntaxin16 (Sadler *et al.*, 2015). Of

interest, we were unable to demonstrate coimmunoprecipitation of Syntaxin4 with VAMP3 in response to insulin at times of exposure of insulin ranging from 3 to 60 min (unpublished data), suggesting that the delivery of GLUT4 to the cell surface uses the same SNARE machinery at all time points.

DISCUSSION

Studies have reported that siRNA-mediated depletion of VAMP2 results in abrogated insulin-stimulated GLUT4 translocation to the plasma membrane of adipocytes, L6 muscle cells, and HL-1 cardiomyocytes, and VAMP5 was also shown to mediate insulin-stimulated GLUT4 translocation (Randhawa *et al.*, 2004; Williams and Pessin, 2008; Kawaguchi *et al.*, 2010; Schwenk *et al.*, 2010). Similarly, studies using either inhibitory peptides or expression of SNARE protein domains have supported a role for VAMP2 in insulin-stimulated GLUT4 translocation, with VAMP3 mediating translocation by other stimuli, such as osmotic shock, GTP γ S, or valinomycin; VAMP7 has also been reported to mediate GLUT4 translocation in response to osmotic shock (Millar *et al.*, 1999; Williams and Pessin, 2008).

Paradoxically, other studies have suggested that many SNARE-mediated trafficking events use multiple v-SNAREs (e.g., Liu and Barlowe, 2002), suggesting functional redundancy at the level of VAMPs (Yang *et al.*, 1999). In support of this model, Zhao *et al.* (2009) showed that VAMP2, 3, or 8 could each separately restore insulin-stimulated GLUT4 translocation in cells devoid of endogenous VAMP2, 3, or 8. Such data led these authors to propose that these VAMPs could operate as v-SNAREs for GLUT4 translocation interchangeably. Consistent with this notion, we show here that all of the VAMPs expressed in 3T3-L1 adipocytes are capable of forming an SDS-resistant SNARE complex with Syntaxin4 and SNAP23. It should be noted, however, that others have reported that VAMP3 cannot form a fusogenic pairing with Sx4/SNAP23 but does so with Sx4/SNAP25 (Hu *et al.*, 2007). Studies in which one or more VAMP isoform is absent (through either genetic or siRNA-mediated means) raise the possibility that other trafficking routes can compensate and deliver GLUT4 to the cell surface by a distinct route. A prerequisite of this model is that VAMPs are sorted into distinct compartments. Zhao *et al.* (2009) argued this is not the case, based on colocalization of different VAMPs and GLUT4 in isolated membrane fractions stained with anti-VAMP antibodies, as between 20 and 50% overlap was observed between VAMP2, VAMP3, VAMP8, and GLUT4. One problem with this approach is that a vesicle with quite different levels of VAMP-x and VAMP-y will be similarly decorated with fluorescent antibodies, making functional distinction between these overlapping fractions difficult. The analysis may also be skewed if one or more of the VAMPs are present at very different levels of expression.

To address these and other issues, we examined all the VAMPs expressed in 3T3-L1 adipocytes in a systematic manner, avoiding knockdown or genetic deletion, to prevent counterregulatory mechanisms overly complicating our analysis. Our first striking observation is that of all the VAMPs, only VAMPs 2–4 exhibit up-regulation in the fibroblast-to-adipocyte conversion. Although this is not an *a priori* reason to rule out any protein playing a functional role in the insulin response, several proteins that are involved in insulin-stimulated GLUT4 translocation are up-regulated during differentiation (El-Jack *et al.*, 1999; Shewan *et al.*, 2003; Shi *et al.*, 2008). Quantification of the absolute levels of VAMPs provides further insight. VAMP8 and VAMP3 are the most abundant isoforms in adipocytes, being expressed at approximately twofold and fourfold higher levels than VAMP2, respectively. VAMP4 is the next most abundant; this VAMP has been implicated in GLUT4 sorting but not exocytosis

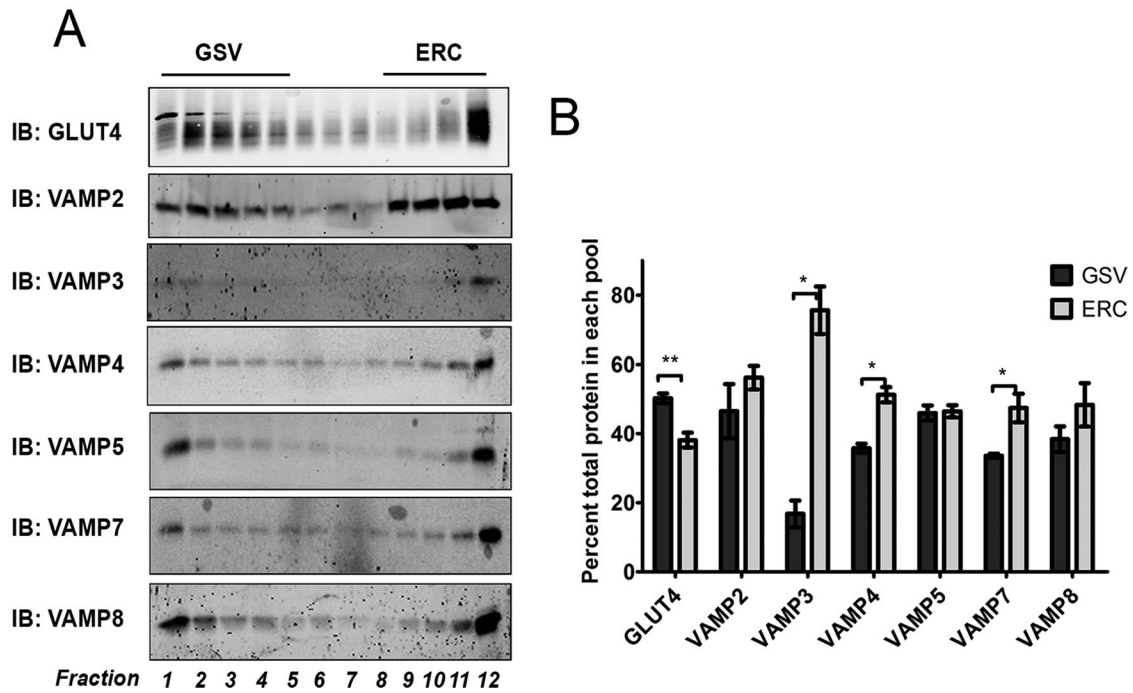


FIGURE 5: VAMP isoforms are differentially distributed on iodixanol gradients. (A) LDM fractions from serum-starved (basal) 3T3-L1 adipocytes were resuspended in HES buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA) and centrifuged on self-forming iodixanol gradients as described. Twelve equal-volume fractions were collected and subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. Fractions were loaded in order of decreasing density; fractions 1–5 represent GSV-enriched fractions, and 9–12 represent ERCs (Hashiramoto and James, 2000). (A) Immunoblots from a typical experiment and (B) the mean values \pm SD for percentage of total protein across all fractions found in the GSV and ERC pools from three separate experiments; values were compared using Student's *t* test, **p* < 0.05, ***p* < 0.01.

and is expressed at levels approaching those measured for VAMP2. By contrast, VAMP5 and VAMP7 are weakly expressed in these cells. High levels of VAMP3 and VAMP8, known to function in endosomal trafficking, are consistent with the high secretory capacity of fat cells that are known to secrete many adipocytokines. This may also explain why either VAMP can restore insulin-stimulated GLUT4 translocation (in cells devoid of VAMPs 2, 3, and 8), as a significant fraction of GLUT4 is delivered to the cell surface from endosomes; in 3T3-L1 adipocytes, up to 40% of GLUT4 is present within transferrin receptor-positive compartments (Livingstone *et al.*, 1996).

Of interest, we observe different patterns of localization and insulin responsiveness of these different VAMP isoforms. As previously reported, insulin stimulates the delivery of VAMP2 and VAMP3 to the plasma membrane; this is consistent with the notion that insulin triggers delivery of VAMP2-positive GSVs and VAMP3-positive endosomes to the cell surface and that both of these compartments contain GLUT4 (Volchuk *et al.*, 1995; Livingstone *et al.*, 1996; Olson *et al.*, 1997). We did not observe insulin-stimulated delivery of endogenous VAMP8 to the cell surface, a key argument against a functional role for this VAMP in normal adipocyte physiology. When LDM fractions were subfractionated into GSV-enriched and endosome-enriched fractions, all VAMPs were present in both fractions, with the notable and consistent exception of VAMP3, which was predominantly localized within the endosomal fraction. These data, together with the lack of an effect of insulin on VAMP8, strongly suggest that VAMP2 is the main v-SNARE for GSV delivery to the cell surface. This data are supported by coimmunoprecipitation of VAMP2 but no other VAMP with Syntaxin4 (with the exception of VAMP5, which is known to interact with Syntaxin4 in cardiomyocytes; Schwenk *et al.*, 2010).

A combination of the quantification and subcellular fraction data presented here allows us to make some approximations regarding the distribution of VAMP isoforms on GLUT4 vesicles. VAMP2 is expressed at 8.6×10^5 copies/cell and VAMP3 at 1.5×10^6 copies/cell. Sixty percent of these proteins are localized within the LDM fraction (Figure 4) comprising both GSVs and endosomal fractions (Figure 5). VAMP2 is roughly equally split between these two fractions, but VAMP3 is mainly localized within the endosomal fraction (80%; Figure 5). Hence, within the GSV-enriched fraction, there are 241,000 copies of VAMP2 and 133,000 copies of VAMP3; in the endosomal fraction, there are 2.5-fold more copies of VAMP3 than VAMP2. Using a value of 2.8×10^5 copies of GLUT4 per cell (Calderhead *et al.*, 1990) and the data in Figure 5 to distribute this GLUT4 between the GSV and endosomal fractions, we estimate that GSVs contain 4 molecules of VAMP2 and 2 of VAMP per molecule of GLUT4; Kupriyanova *et al.* (2002) estimated that one GSV contains ~ 4 molecules of GLUT4; using this estimate, our data would equate to 16 VAMP2 molecules per GSV, compared with 8 copies of VAMP3. These data compare favorably with the estimates of the number of SNAREs required to catalyze fusion (Weninger *et al.*, 2003; Zhang *et al.*, 2004; Sudhof and Rothman, 2009; Wiederhold and Fasshauer, 2009) but are considerably lower than the estimates of VAMP2 levels on isolated synaptic vesicles (Takamori *et al.*, 2006). This may reflect both biological differences in terms of the kinetics of the secretion event in question (neuronal exocytosis is faster than GLUT4 delivery to the cell surface) and clear oversimplification in our analysis, as it is unlikely that the distributions of these different proteins in the GSV and endosomal fractions are uniform. Nonetheless, the data are consistent with VAMP2 being the major VAMP involved in GSV fusion with the cell surface.

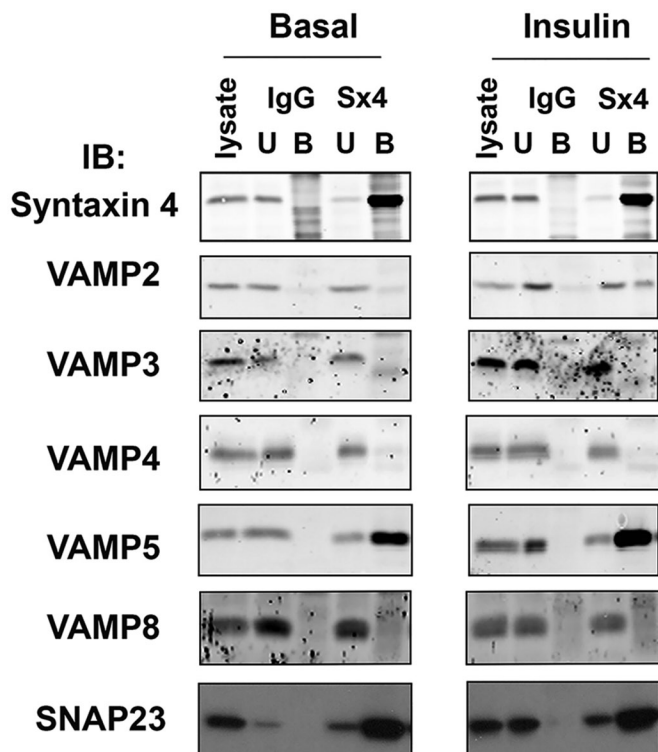


FIGURE 6: VAMP2 coimmunoprecipitates with Syntaxin4 in adipocytes. A 5 μ l amount of anti-Syntaxin4 (Sx4) or random rabbit immunoglobulin G (IgG) was used to immunoprecipitate from 1.5 mg of lysate prepared from 3T3-L1 adipocytes incubated without (Basal) or with 1 μ M insulin (Insulin) for 30 min as described. Immunoprecipitated proteins were recovered by centrifugation, washed, and eluted in 2 \times LSB. Samples were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Data from a typical experiment, repeated at least three times with qualitatively similar results; for each immunoprecipitation, the basal and insulin-stimulated samples were run on the same gel with other samples, which have been omitted here for clarity. Lysate, the starting cell lysate; U, material remaining in the lysate after the immunoprecipitation; B, the immunoprecipitated material.

In sum, we provide the first detailed analysis of the cellular content of VAMP isoforms in a single cell type and show that each of these VAMPs can form an SDS-resistant ternary complex with Syntaxin4 and SNAP23. Our data support studies that suggested an important role for VAMP2 in GSV fusion with the plasma membrane and argue against the notion of plasticity in the context of normal cell function. We do not dispute the data presented by Zhao *et al.* (2009) showing that in the absence of VAMPs 2, 3, and 8, any of these VAMPs alone can reconstitute insulin-stimulated GLUT4 delivery to the cell surface. However, our data are consistent with the notion that in cells not devoid of any VAMP, VAMP2 is the most likely candidate v-SNARE for GLUT4 delivery to the cell surface.

MATERIALS AND METHODS

Cells, antibodies, and plasmid constructs

3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and grown and differentiated into adipocytes as outlined (Roccisana *et al.*, 2013). All anti-VAMP antibodies were from Synaptic Systems (Göttingen, Germany), anti-SNAP23 and anti-Syntaxin4 were from Abcam (Cambridge,

United Kingdom), and all other antibodies were as described (Roccisana *et al.*, 2013). GST-VAMPs 2 and 3 were as described (Millar *et al.*, 1999; Brandie *et al.*, 2008). GST-VAMPs 4, 5, 7, and 8 were from Andrew Peden (University of Sheffield, Sheffield, United Kingdom).

Recombinant protein expression and purification and SNARE complex assembly assays

Glutathione S-transferase (GST)-tagged VAMP proteins were expressed in BL21(DE3) cells and purified as described (Brandie *et al.*, 2008). Syntaxin4 and SNAP23 were purified exactly as outlined in (Aran *et al.*, 2009), and all proteins were dialyzed against phosphate-buffered saline (PBS) and stored at -80°C until use. For SNARE complex assembly assays, equimolar (~ 4 μ M) amounts of GST-VAMP protein, histidine (His)-SNAP23, and thrombin-cleaved Syntaxin4 were combined in a volume of 500 μ l of PBS supplemented with 100 μ g/ml bovine serum albumin and incubated for the indicated time at 4°C with rotation. Reactions were stopped by the addition of 5 \times Laemmli sample buffer (LSB; 300 mM Tris-Cl, pH 6.8, 10% SDS, 50% glycerol, 25% β -mercaptoethanol, 0.05% bromophenol blue) and immediately heated to 95°C for 5 min. Samples were subjected to SDS-PAGE and immunoblot analysis on the same day as the experiment.

Immunoprecipitation

3T3-L1 adipocytes were treated with 1 mM *N*-ethylmaleimide and then lysed in immunoprecipitation buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 150 mM NaCl, 2 mM β -glycerophosphate, 1 mM dithiothreitol, 1% [vol/vol] Triton X-100, and protease inhibitors) and centrifuged at $12,500 \times g$ for 20 min at 4°C . A 1.5-mg amount of lysate was precleared using protein A beads and then incubated with anti-Syntaxin4 or random rabbit serum for 2 h on ice. Protein A beads were added for a further 2 h and then separated from unbound material by brief centrifugation. Unbound material was retained for analysis and bound material washed three times and eluted using 2 \times LSB (Sadler *et al.*, 2015).

Subcellular fractionation and immunoblotting

Subcellular fractionation into plasma membrane, high- and low-density membranes, and cytosolic fractions was performed using a well-characterized procedure (Piper *et al.*, 1991; Roccisana *et al.*, 2013); this method routinely underestimates the effect of insulin on GLUT4 translocation to the plasma membrane (Piper *et al.*, 1991; El-Jack *et al.*, 1999; Hashiramoto *et al.*, 2000). Iodixanol gradient centrifugation analysis of freshly isolated LDM fractions was performed as described (Hashiramoto and James, 2000; Maier and Gould, 2000). To quantify the fraction of GLUT4 within the GSV fractions, we expressed the immunoblot signal derived from lanes 1–5 as a fraction of the total GLUT4 signal across all fractions. Total membranes were prepared as described (Roccisana *et al.*, 2013). SDS-PAGE and immunoblotting were performed as described (Roccisana *et al.*, 2013). Immunoblot signals were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

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